

AD-A272 135



## NOTATION PAGE

Form Approved  
OMB No. 0704-0188

1a. REF UNC		1b. RESTRICTIVE MARKINGS WR-157-92	
2a. SECURITY CLASSIFICATION AUTHORITY Unclassified		3. DISTRIBUTION / AVAILABILITY OF REPORT (2)	
2b. DECLASSIFICATION / DOWNGRADING SCHEDULE		5. MONITORING ORGANIZATION REPORT NUMBER(S)	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)		7. NAME OF MONITORING ORGANIZATION DTIC ELECTE NOV 09 1993 A	
6a. NAME OF PERFORMING ORGANIZATION Division of Biochemistry	6b. OFFICE SYMBOL (If applicable)	8. ADDRESS (City, State, and ZIP Code) Walter Reed Army Institute of Research Washington, DC 20307-5100	
8a. NAME OF FUNDING / SPONSORING ORGANIZATION U.S. Army Medical Research and Development Command	8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) Fort Detrick, Frederick, MD 21701		10. SOURCE OF FUNDING NUMBERS	
		PROGRAM ELEMENT NO.	PROJECT NO.
		TASK NO.	WORK UNIT ACCESSION NO.
11. TITLE (Include Security Classification) Preparation and Use of Liposomes in Immunological Studies			
12. PERSONAL AUTHOR(S) Carl R. Alving, Shigeki Shichijo, Inger Mattsby-Baltzer, Roberta L. Richards, and Nabila M. Wassef			
13a. TYPE OF REPORT	13b. TIME COVERED FROM _____ TO _____	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
16. SUPPLEMENTARY NOTATION			
17. COSATI CODES		18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
		Liposomes, lipid A, vaccines	
19. ABSTRACT (Continue on reverse if necessary and identify by block number)			
<p>Methods are described for preparation of liposomes for use in immunological assays or for immunization; for complement-dependent immune lysis of liposomes assay; for purification of antibodies by affinity binding to liposomes; for preparation of lipid A and insertion of lipid A into liposomes; and for use of liposomes for immunization.</p>			
20. DISTRIBUTION / AVAILABILITY OF ABSTRACT <input type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT. <input type="checkbox"/> DTIC USERS		21. ABSTRACT SECURITY CLASSIFICATION	
22a. NAME OF RESPONSIBLE INDIVIDUAL		22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL

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# **Liposome Technology**

## **2nd Edition**

### **Volume III**

## **Interactions of Liposomes with the Biological Milieu**

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CRC Press  
Boca Raton Ann Arbor London Tokyo

93 11 5 098

**Library of Congress Cataloging-in-Publication Data**

Liposome technology / editor, Gregory Gregoriadis. — 2nd ed.

p. cm.

Includes bibliographical references and indexes.

Contents: v. 1. Liposome preparation and related techniques — v. 2. Entrapment of drugs and other materials — v. 3. Interactions of liposomes with the biological milieu.

ISBN 0-8493-6707-7 (v. 1). — ISBN 0-8493-6708-5 (v. 2). — ISBN 0-8493-6709-3 (v. 3)

1. Liposomes. 2. Drug targeting. 3. Drugs — Vehicles.

I. Gregoriadis, Gregory.

RS201.L55L53 1992

615'.19—dc20

92-8975

CIP

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Direct all inquiries to CRC Press, Inc., 2000 Corporate Blvd., N.W., Boca Raton, Florida, 33431.

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International Standard Book Number 0-8493-6707-7 (Volume I)

International Standard Book Number 0-8493-6708-5 (Volume II)

International Standard Book Number 0-8493-6709-3 (Volume III)

Library of Congress Card Number 92-8975

Printed in the United States of America 1 2 3 4 5 6 7 8 9 0

Printed on acid-free paper

## Chapter 21

**PREPARATION AND USE OF LIPOSOMES IN  
IMMUNOLOGICAL STUDIES**

**Carl R. Alving, Shigeki Shichijo, Inger Mattsby-Baltzer,  
Roberta L. Richards, and Nabila M. Wassef**

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## I. INTRODUCTION

Until recently relatively few immunologists recognized that lipids were antigenic. However, among specialists the lipoidal nature of many commonly studied antigens, including the syphilis-associated antigen (cardiolipin) and Forssman antigen of sheep erythrocytes, was known more than 60 years ago (reviewed in References 1 to 3). The commonly held notion that lipids are poor antigens compared to proteins is both correct and false as a concept. It is correct in that relatively large amounts of lipids are often used for immunization. It is a false concept in that only a fraction of the total weight of the lipid molecule, the polar region, generally accounts for virtually all of the measured antigenic activity, and the polar region can be highly antigenic. For example, the antigenic portion of cardiolipin (CL) accounts for only a fraction (approximately 14%) of the molecular weight of CL, but essentially every case of syphilis, even within a few days of infection by treponemes, results in the production of antibodies against CL.

A major difficulty in studies on lipid immunology in the past was the insoluble nature of lipid antigens. In aqueous suspension lipid antigens formed poorly understood particles, and reactions with antibodies generally were achieved only by adding empirically derived combinations of "auxiliary lipids", mainly lecithin and cholesterol (Chol). The literature in this field is complex and is filled with seemingly bizarre phenomena (reviewed in References 2 and 3). However, the introduction of liposomes allowed the use of concepts of three-dimensional membrane geometry. Physical chemical characteristics of liposomal membranes can now be employed as rational factors

in lipid immunology, and therefore we like to think that "the invention of liposomes thus has turned the problem of insolubility of lipids into a virtue."<sup>3</sup>

Liposome immunology started with studies on liposomes as targets of antibody binding and complement damage, and as models for complement-mediated membrane damage (reviewed in References 3 and 4). Membrane damage causes permeability of liposomes to relatively large molecules. Measurement of permeability is a useful method for assaying activities of antibodies against glycolipids, phospholipids, and liposome-associated proteins, and for studying immune and nonimmune complement-mediated membrane damage. It is also useful for detecting antigenic activity of known or unknown lipid antigens. The glucose release method often used by us was originally invented by Kinsky<sup>5,6</sup> and the version described here is a modified one that uses the same basic format.

The wide popularity of liposomes has been due in part to the flexibility of the system and to the large variety of potential applications. In the immunology domain, aside from studying the mechanism of complement activation and complement-induced membrane damage, liposomes have been employed by us for analysis of antibodies against numerous antigens, including neutral glycolipids, gangliosides, lipid A, liposomes themselves (mainly the phospholipid portion), and liposome-bound protein antigen. We have also used liposomes as carriers of antigens and as adjuvants.<sup>7</sup> With liposomes we have immunized mice, rabbits, monkeys, or humans with a variety of antigens, including cholera toxin,<sup>8,9</sup> malaria antigens,<sup>9,13</sup> and even a small unconjugated peptide.<sup>14</sup> We have produced "antiliposome" antibodies in rabbits and mice,<sup>15-17</sup> and we have even produced monoclonal antiliposome antibodies against liposomal phospholipids, and Chol.<sup>18-22</sup> Immunization procedures generally involve only a single or a few intravenous (i.v.), intramuscular (i.m.) or subcutaneous (s.c.) injections. An advantage to immunization with liposomes is that the same liposomes that are used for immunization can be used for assaying the antibodies that are produced. In this chapter, we shall describe the methods for preparing liposomes both for assay of antibodies and for use as carriers of antigens for immunization.

In setting up a system for assaying immune damage to liposomes, how does one select an appropriate antigen? The first major decision is whether to use a lipid, such as a phospholipid, a glycolipid, or lipid A (or lipopolysaccharide), or whether to use a surface-bound protein, such as cholera toxin bound to liposomal G<sub>M1</sub>, or even a hydrophobic protein inserted in the lipid bilayer. Each antigen in liposomes has its own peculiar characteristics, and each has its own rewards and difficulties. No liposomal antigen is inherently better than the others. In our experience, the "simplest" antigen is the lipids (mainly the phospholipids) that are used in preparing the liposomes themselves. Whichever antigen is employed, an appropriate antiserum must be obtained, and this can sometimes be a difficult factor.

In addition to using liposomes to assay antibodies, the same liposomes can be used to purify antibodies by elution from the liposomes. We call this

technique "affinity binding to liposomes",<sup>23</sup> and the method has been used for purifying a variety of antiglycolipid antibodies.<sup>24-28</sup>

Although assays of complement-dependent permeability of liposomes have dominated the field, such assays do not measure noncomplement-fixing antibodies. Partly because of this, agglutination of liposomes is sometimes employed (reviewed in Reference 4). As an offshoot of the technique of immunoagglutination, we have prepared liposomes in the LA or LAC (liposome-antibody or liposome-antibody-complement) state.<sup>29</sup> The LA and LAC liposomes are washed free of unbound serum constituents (see Section III) and are useful for certain specialized applications such as study of immune phagocytosis.<sup>30-32</sup>

## II. COMPLEMENT-DEPENDENT IMMUNE LYSIS OF LIPOSOMES

### A. MATERIALS

#### 1. Phospholipids and Other Lipids

Dimyristoyl phosphatidylcholine (DMPC) is available from a variety of commercial suppliers, including Calbiochem-Behring (San Diego, CA), Sigma Chemical Co. (St. Louis, MO), Avanti (Alabaster, AL), and PL Biochemicals (Milwaukee, WI). We now obtain DMPC and dimyristoyl phosphatidylglycerol (DMPG) exclusively from Avanti due to the superior purity of their synthetic phospholipids. DMPC and DMPG from other sources have been found by us to contain variable amounts of lysophospholipids, which are highly undesirable in liposomes used for immunization. Most phospholipids from natural sources, such as egg or soybean phosphatidylcholine (PC), brain phosphatidylserine (PS), brain phosphatidic acid (PA), brain or plant phosphatidylinositol (PI), heart CL, and plant or bacterial phosphatidylethanolamine (PE) are not used by us as the primary phosphatide, except for special purposes, because of the instability and leakiness of the resulting liposomes.

Chol is available from numerous commercial sources. Because of oxidation products that inevitably form even in unopened bottles, recrystallization of Chol has been recommended for certain applications.<sup>33</sup> Recrystallized Chol is used by us in liposomes for immunization in order to avoid the possibility of toxicity due to oxidation products. In our experience, for an unknown reason liposomes containing recrystallized Chol sometimes clump and aggregate into cordlike structures upon standing, and because of this we generally do not recrystallize Chol for liposome lysis assays. Dicetyl phosphate (DCP) is available from K and K Laboratories (Fairview, NJ), Sigma Chemical Co. (St. Louis, MO), or PL Biochemicals (Milwaukee, WI).

#### 2. Glycolipid Antigens

Some of the current commercial sources of glycolipids that we employ are as follows: galactocerebroside (beef brain), Matreya (formerly Supelco,

Pleasant Gap, PA), Sigma, and PL Biochemicals; lactocerebroside, Calbiochem, Matreya; ceramide trihexoside (human erythrocyte), Matreya; ceramide tetrahexoside (globoside) (human erythrocyte), Matreya, Sigma; digalactosyl diglyceride (plant), Matreya, Sigma, and PL Biochemicals; gangliosides (mixed) beef brain, Calbiochem, Matreya, Sigma, and PL Biochemicals; gangliosides  $G_{M1}$ ,  $G_{D1a}$ , and  $G_{D1b}$  (beef brain), Matreya, Calbiochem.

### 3. Chloroform and Other Materials

Chloroform can be obtained in a variety of grades. Chloroform (even reagent grade) inevitably deteriorates upon sitting in the bottle. *Solubilization of lipids as stock solutions in chloroform in which degradation products of chloroform are present can kill the subsequent liposome immune lysis assay.* At 1- to 3-month intervals, we redistill the chloroform used for formulating liposomes. This procedure is highly recommended. After distillation one should add 0.7% ethanol as a preservative.

Pear-shaped boiling flasks are manufactured by Kimball (Kimax) and Corning (Pyrex®) and are readily available from many vendors. However, in our opinion, the flasks marketed independently by Lurex Scientific Inc., Vineland, NJ, (1982 catalog number JM-5490) have the best shapes.

Microbeads (approximately 0.5 mm, class VA, -30 + 35) can be obtained from Cataphote Division of Ferro Corp, Cleveland, OH and Jackson, MS). Before using, the beads are washed by soaking for several days in concentrated  $H_2SO_4$ , rinsed to neutral pH with daily changes of water for a week, and dried thoroughly in a 110°C oven.

### 4. Glucose Assay Reagent

The buffer consists of Tris-HCl (100 mM, pH 7.5) containing 64 mM NaCl, 3.5 mM  $MgCl_2$ , and 0.15 mM  $CaCl_2$ . To this are added the following constituents: hexokinase (yeast) (Boehringer-Mannheim, Indianapolis, IN), 30  $\mu$ g/ml (after dialyzing against water to remove ammonium sulfate, and adding 0.2% sodium azide at a concentration of 50  $\mu$ l per ml of dialyzed enzyme, store at 4°C, and *do not freeze*); glucose-6-phosphate dehydrogenase (yeast) (Boehringer-Mannheim, Grade 1, 15  $\mu$ g/ml (after dialyzing, and adding sodium azide, store at 4°C, and *do not freeze*); ATP, 2 mM (can be stored frozen as a 200-mM stock solution, pH 7.3); NADP, 1 mM (can be stored frozen as a 100-mM stock solution, pH 7.0). Because of potential lability of the ingredients, the glucose assay reagent is prepared from stock solutions only on the day of the experiment.

### 5. Antiserum and Complement

The most potentially frustrating reagent in the liposome lysis system is the antiserum. This is because sera from most animal sources contain numerous "natural" antibodies that react with glycolipids.<sup>24,34</sup> High titer



antiserum against Forssman antigen is commercially available from many vendors in the form of antiserum against sheep erythrocyte serum for complement fixation assays and is known as "hemolysin". However, for antigens that are more readily available than Forssman, high quality antiserum cannot usually be purchased. Fortunately, rabbit antibodies against virtually any simple glycolipid are readily raised.<sup>35</sup> Before bleeding a rabbit, food (but not water) should be withheld at least for 4 h, but overnight is preferable, to prevent the occurrence of lipoidal serum that can interfere with certain assays. When immunizing the rabbit, be certain to obtain a "preimmunization bleeding" to check for natural antibodies either against the glycolipid or against the liposomes themselves (see Section II.B.6). In our experience, natural antibodies against galactocerebroside are unusual, but natural antibodies against lactocerebroside and ceramide trihexoside occur to a varying extent in nearly every normal rabbit serum. Out of dozens of rabbits tested over many years, we have found only one animal that lacked natural antilactocerebroside antibodies and that rabbit was then immunized with lactocerebroside.<sup>34</sup> The most plentiful source of antibodies against many glycolipids is normal rabbit (or even normal human) serum. However, natural antibodies may have specificities that are different from those obtained from immunized animals.<sup>24,34</sup> Purification of antibodies, whether natural antibodies or not, is readily accomplished by the technique of affinity binding to liposomes<sup>23</sup> (see Section III).

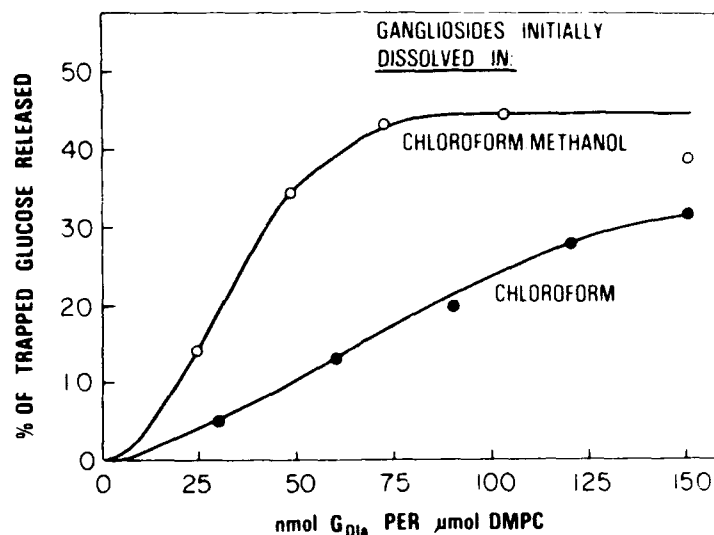
Before using antiserum, it should be heated at 56°C for 30 min to inactivate complement. Fresh serum, either guinea pig or human, is suitable as an added complement source. Although the complement titers of guinea pig and human sera are normally quite different when tested with sheep erythrocytes,<sup>36</sup> they are nearly identical when tested with liposomes.<sup>37</sup> The serum can be frozen indefinitely at -70°C, but it should be aliquoted and should be thawed as rarely as possible. If possible, the sera should be obtained from unanesthetized animals. There is a tendency to use guinea pig serum pooled from numerous animals, but human serum is often taken from a single individual. One should be aware that individual humans or guinea pigs may have natural antiglycolipid antibodies, and this possibility should be considered when deciding whether to use pooled serum or serum from individuals. We routinely test individual guinea pig sera against liposomal glycolipids before selecting a group to pool as a complement source.

Before using any serum, whether antiserum or complement, the serum must be extensively dialyzed to remove endogenous glucose. For complement we routinely dialyze 15 ml of serum twice (1 h each) against 4-l volumes of 0.15 M NaCl.

## **B. PROCEDURE**

### **1. Preparation of Liposomes**

Stock solutions of lipids in chloroform or chloroform/methanol (2/1) are stored at -20°C in graduated cylinders having ground glass tops. Whenever



**FIGURE 1.** Influence of methanol as an initial ganglioside solvent on the effectiveness of the ganglioside as a liposomal antigen. In a pear-shaped flask, ganglioside  $G_{D1a}$ , in the concentrations shown on the abscissa, was dried either from a suspension in chloroform or from solution in chloroform/methanol (2/1). The other liposomal constituents (DMPC:Chol:DCP, 1:0.75:0.11) were then added in chloroform and dried, and the liposomes were swollen and assayed with antiganglioside serum as described in the text.

possible, chloroform is used as the only solvent because it is more readily evaporated than methanol. However with certain lipids, such as gangliosides, much better results are obtained when at least a small amount of methanol is included to solubilize the antigen. Figure 1 shows that it is not enough simply to dry the antigen. The manner in which the antigen is dried can be extremely important.

For most of our studies, the liposome preparation contains phospholipid, Chol, and DCP in molar ratios of 1:0.75:0.11. The rationale for the phospholipid:Chol ratio is that this is the approximate ratio found in lipids of erythrocytes. Whatever proportions are used, the lipids are mixed together in a pear-shaped flask. The volume of the flask should be more than ten times greater than the volume of the anticipated aqueous suspension of liposomes to allow for proper agitation during liposome formation. Using a rotary evaporator, the solvent is removed at approximately 40°C under negative pressure. We use the vacuum obtained from a filter pump aspirator attached to a water faucet. The solvent normally is removed within 2 to 5 min. The flask is then dried further under very low vacuum (<50  $\mu$ m Hg) for 1 h in a dessicator. Even if the dried lipids are allowed to remain under vacuum they can deteriorate, and we discard them after approximately 1 week.

After drying, a small number (approximately 70 to 100  $\mu$ l for a 10-ml pear-shaped flask) of 0.5-mm glass beads are added. An appropriate volume

of "swelling solution" (0.308 *M* glucose), amounting to the final desired volume of liposome suspension is added (the volume of the lipids themselves is considered negligible). In most of our experiments, the amount of swelling solution is dictated by the amount of liposomal phospholipid: the phospholipid is always 10 mM with respect to the swelling solution. The flask is closed with a rubber stopper that has been covered with Parafilm, and the flask is vortexed vigorously for 1 to 2 min. (or more, if necessary). There is some art involved in the vortexing. With a little practice, the glucose and beads can be made either to swirl rapidly or to bounce vigorously in the flask, and both of these processes help to disperse the lipid. Care is taken to disperse all of the dried lipid from the flask wall.

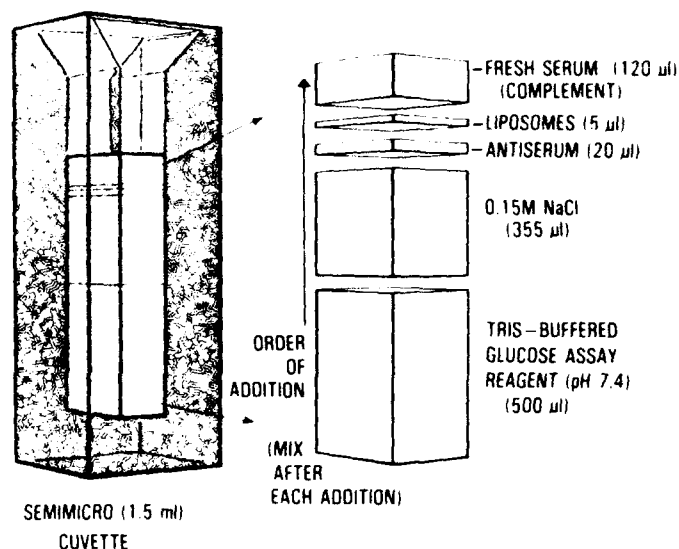
## 2. Removal of Untrapped Glucose

The lipid suspension (liposomes) that results from the above procedure is a multilamellar vesicle (MLV) preparation. Before using the liposomes, untrapped (i.e., unencapsulated) glucose must be removed. The easiest methods to achieve this are dialysis (for example, 0.2 ml of liposomes dialyzed in 6-mm cellulose tubing for 1.5 h against 250 ml of 0.15 *M* NaCl) or several washes by centrifugation (for example, 0.2 ml of liposomes diluted with 10 ml of 0.15 *M* NaCl are centrifuged at approximately  $27,000 \times g$  for 10 min at 20°C, and 0.15 *M* NaCl is used for resuspension of the pellet).

The centrifugation procedure for initial washing of liposomes risks the loss of small liposomes that lack sufficient density to form a pellet. *However, when certain gangliosides are used in liposomes the centrifugation procedure is strongly recommended.* Liposomes containing gangliosides  $G_{M1a}$  or  $G_{T1b}$  release substantially more glucose due to immune damage when they are washed initially by centrifugation rather than dialyzed to remove untrapped glucose. The reason for this phenomenon is not fully understood, but it probably does not apply to most neutral glycolipids, or even to many other gangliosides, such as  $G_{M1}$  or  $G_{D1b}$ .

## 3. Immune Lysis Assay

After swelling the liposomes in glucose and removal of most of the untrapped glucose, the liposomes are ready for use in immunological assays. The contents of a typical assay cuvette are shown in Figure 2. The total volume of 1 ml always includes 0.5 ml of Tris-buffered glucose assay reagent. The other individual ingredients, including liposomes (which can be added directly on shaved-down feet of "plumpers" that double as mixers, available from Calbiochem, San Diego, CA), antiserum, complement, and 0.15 *M* NaCl, may be added in different proportions, as needed, in a total volume of 0.5 ml. When materials are in short supply, the volumes can be scaled down to 60% of the volumes described here. Although complement is added last in the cuvette shown in Figure 2, one could add either antiserum or liposomes last if that were more convenient. Usually the assay is performed



**FIGURE 2.** Contents of a typical assay cuvette for measuring complement-dependent immune damage to liposomes by the glucose release method.

at room temperature, but the temperature should not be less than 20°C (also, see Notes of Caution regarding temperature, in Section II.B.6).

For assaying release of trapped glucose due to immune damage to liposomes, two  $A_{340}$  readings are required, namely immediately before, and 30 min after adding the last ingredient (either antiserum, liposomes, or complement). The final  $A_{340}$  is then corrected by subtracting the  $A_{340}$  due to the last ingredient. The correction value for the last ingredient is determined independently in a separate assay cuvette containing only that variable. Usually there is no requirement for adding a correction due to changes of light scattering of "lysed" liposomes. This is because the liposomes simply become "leaky", and they do not "burst" like erythrocytes and release molecules that influence absorbance. However, occasionally light-scattering changes do occur, for example, due to agglutination. If there is any question, check for light-scattering effects with "incomplete assay reagent" (see below).

The  $A_{340}$  should be linearly related to the amount of glucose released. However, the absolute glucose concentration is only rarely calculated, and glucose release is usually expressed as "percent of trapped glucose released". For this calculation, the amount of glucose that was initially trapped must be assayed.

#### 4. Assay of Initially Trapped Glucose

There are two steps required for this assay: (1) measurement of "total glucose" (i.e., a total combination of both the trapped glucose and any residual

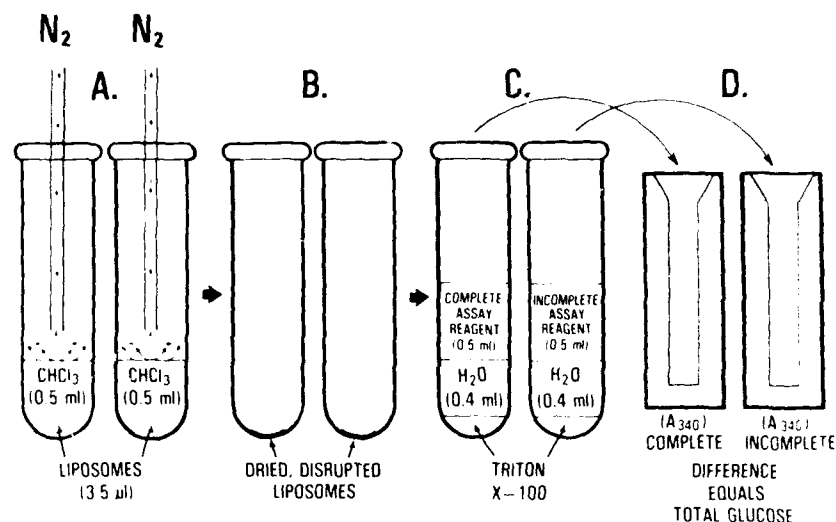


FIGURE 3. Measurement of total (trapped + untrapped) liposomal glucose.

untrapped glucose), and (2) measurement of untrapped glucose. The trapped glucose is then calculated by subtracting untrapped glucose from the total.

The procedure for measuring "total glucose" is summarized in Figure 3. An appropriate aliquot of liposomes (3 to 5  $\mu$ l) is added to the bottom of each of two 1.5 cm  $\times$  15 cm test tubes, and 0.5 ml of chloroform is added as a dissolving agent to each tube (Figure 3A). The chloroform is removed under a stream of  $N_2$  (Figure 3B) or by evaporation under vacuum in a Speed Vac Concentrator (Savant, Hicksville, NY) when there are a large number of samples, then 0.1 ml of 10% Triton® X-100 in 0.1 M Tris-HCl (pH 8) is added, and the tubes are vigorously shaken on a vortex mixer. Then 0.4 ml of water are added and followed by vortexing. To one of the tubes glucose assay reagent (referred to as "complete" reagent) (0.5 ml) is added, and to the other tube is added "incomplete" reagent that cannot detect glucose because it is deficient in one or more of the assay ingredients (Figure 3C). Incomplete reagent may either lack all enzymes and cofactors and contain only Tris buffer, or it may lack only one critical constituent, such as NADP. Approximately 10 min after the final addition, the tube contents are transferred to cuvettes for measurement of  $A_{340}$  (Figure 3D). Blank readings of  $A_{340}$  of the same cuvettes, with the cuvettes containing only water, should be used as correction factors. The  $\Delta A_{340}$  (complete - incomplete) gives the  $A_{340}$  that corresponds to the total glucose.

Untrapped glucose is readily assayed directly in two cuvettes. The cuvettes initially each contain 0.5 ml of assay reagent, complete reagent in one and incomplete reagent in the other, and 0.495 ml of 0.15 M NaCl.  $A_{340}$  is then measured, 5  $\mu$ l of liposomes is added and  $A_{340}$  is measured again. As sum-

**TABLE 1**  
**Protocol and Analysis of Experimental Variables for Assaying**  
**Untrapped Glucose**

Addition or measurement	Cuvette containing		Variables measured <sup>a</sup>
	Cuvette containing complete reagent (measures glucose)	incomplete reagent (cannot measure glucose)	
Assay reagent			
Complete	500 $\mu$ l		
Incomplete		500 $\mu$ l	
Measure $A_{340}$	$A^b$	$B^b$	$V_1 + V_2$ $V_3 + V_4$
	$\Delta A_{340} = A - B = C^b$		$V_1 + V_3 - V_2 - V_4$
Liposomes	5 $\mu$ l	5 $\mu$ l	
Measure $A_{340}$ after 10 min	$D^b$	$E^b$	$V_1 + V_3 + V_5 + V_6$ $V_2 + V_4 + V_5$
	$\Delta A_{340} = D - E = F^b$		$V_1 + V_3 + V_5 + V_6$ $- V_2 - V_4 - V_5$
			$= V_1 + V_3 + V_6 - V_2 - V_4$
	$\Delta A_{340} = F - C = \text{untrapped glucose}$		$V_1 + V_3 + V_6$ $- V_2 - V_4 - V_1 - V_3$ $+ V_2 + V_4$ $= V_6$

<sup>a</sup> Variables measured:  $V_1$ ,  $A_{340}$  inherent to complete reagent cuvette (scratches, etc.);  $V_2$ ,  $A_{340}$  inherent to incomplete reagent cuvette;  $V_3$ ,  $A_{340}$  of complete reagent;  $V_4$ ,  $A_{340}$  of incomplete reagent;  $V_5$ ,  $A_{340}$  due to light scattering of liposomes;  $V_6$ ,  $A_{340}$  due to untrapped glucose.

<sup>b</sup> A, B, D, and E represent the actual measurements (data) obtained in this assay, while C and F represent values derived from calculations, as shown.

marized in Table 1, the untrapped glucose is determined by the following equation:

(complete  $A_{340}$  - incomplete  $A_{340}$ ) after liposomes - (complete  $A_{340}$  - incomplete  $A_{340}$ ) before liposomes

This calculation compensates for all of the variables measured in the system (see Table 1 for details of the variables that are measured).

### 5. Advantages and Disadvantages of the Assay

The glucose release immune assay is convenient, quite sensitive (for example, it can easily measure 3 to 5 nmol of released glucose), reliable, and after investment in the hardware (mainly the spectrophotometer and cuvettes), inexpensive. In our opinion it is superior to radioisotope methods because the latter methods require inconvenient separation of released isotope, and the separation techniques usually eliminate any potential benefit of greater sensitivity. Although fluorimetric methods (e.g., release of quenched

fluorescent marker or fluorogenic enzyme substrate) are probably equivalent in terms of convenience and are certainly more sensitive, they are not necessarily less expensive, and they must cope with their own unique corrections and controls.<sup>38</sup> Fluorometers also are less widely available than spectrophotometers as general laboratory equipment.

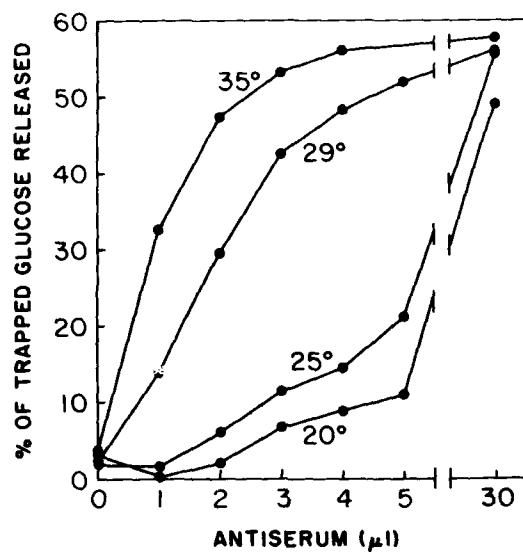
The major disadvantage of the glucose release method when compared to other liposome lysis assays is the presence of high levels of endogenous glucose in all sera, and frequently even in culture fluid sources. This often necessitates dialysis of numerous sera. We routinely accomplish this adequately, when necessary, by attaching as many as 50 to 60 dialysis sacs (containing 1 ml of antiserum each) by paper clips around the wall of an 18-l jar containing 0.15 M NaCl.

The major disadvantage (or advantage, depending on one's viewpoint) of all liposome immune lysis assays is that they measure only complement-fixing antibodies. However, the "resolution" of the liposomal glucose release method in being able to detect a positive, but low-level, antibody activity is such that it probably exceeds most other available techniques for detecting antibodies against lipids and other hydrophobic antigens.

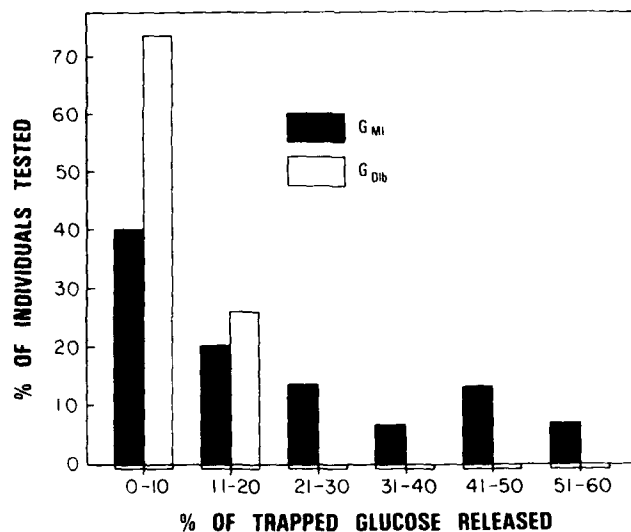
## 6. Three Notes of Caution

(1) The liposomal immune lysis assay described above usually is performed at room temperature (22 to 24°C). However, it is important to recognize that the system can be extremely sensitive to temperature. Temperature effects (described in detail in Reference 39) can influence complement titer, antibody titer, degree of antigen expression, membrane fluidity, and molecular orientation of membrane lipids. Below 20°C, the assay system loses activity, probably due to multiple factors. Figure 4 shows dramatic effects of temperature that were observed during assays of the antibody titer of an anti-galactocerebroside serum. In the experiment shown, when excess antiserum (30  $\mu$ l) was added, the temperature effects were not as evident. Effects such as those in Figure 4 do not occur with every antibody or antiserum, but when they do occur they can be devastating. We have had the disconcerting experience of apparently "losing" the activity of a monoclonal antiphospholipid antibody between summer and winter. The activity was "regained" by performing the reactions in tubes (instead of cuvettes) in a 35 to 37°C water bath, and measuring  $A_{340}$  after transferring the tube contents to cuvettes at room temperature.

(2) The experimenter should be aware that all sources of serum contain naturally occurring antibodies against numerous glycolipids,<sup>24,34</sup> phospholipids,<sup>40</sup> and Cholesterol.<sup>22</sup> Occurrence of antibodies against particular glycolipids or phospholipids varies between species, among individuals in a given species, and even among groups, such as old or young animals. As an example, Figure 5 shows that normal guinea pig serum (which is routinely used as a complement source) may or may not contain natural antibodies against



**FIGURE 4.** Influence of temperature on antibody titer against galactocerebroside as a liposomal antigen. The liposomes contained DMPC/Chol/DCP (1:0.75:0.11) plus 150  $\mu$ g of galactocerebroside per  $\mu$ mol of PC. (From Alving, C. R., Urban, K. A., and Richards, R. L., *Biochim. Biophys. Acta*, 600, 117, 1980. With permission.)



**FIGURE 5.** Prevalence of naturally occurring antibodies against gangliosides G<sub>M1</sub> and G<sub>D1b</sub> in sera from 30 normal guinea pigs. Liposomes consisted of DMPC/Chol/DCP/ganglioside (1:0.75:0.11:0.1). In each assay, 67  $\mu$ l of fresh (unheated) guinea pig serum served both as antibody and complement source.





ganglioside G<sub>M1</sub> or G<sub>D1b</sub>, and the occurrence of such natural antibodies depends on the individual animal.

(3) In view of the increased antibody titer that is often seen at 35°C (Figure 4), there might be an inclination by the experimenter to run all assays at 35 to 37°C. However, one should also recognize that naturally occurring antiglycolipid or antiphospholipid antibody activity in guinea pig complement (or in antiserum) may be strongly active at 35 to 37°C, but it might not be expressed at room temperature (Figure 6). Such naturally occurring background antibody activity in the complement may interfere with, or confuse the interpretation of, the immune assay at 35 to 37°C, but often there may be no problem at 22 to 25°C.

### III. PURIFICATION OF ANTIBODIES BY AFFINITY BINDING TO LIPOSOMES

#### A. BACKGROUND

The utility of liposomes as targets for antibody- and complement-mediated lytic activity is strongly influenced by the quality of the antibody. Discovery of the widespread occurrence of naturally occurring antibodies against glycolipids and phospholipids<sup>24,34,40</sup> (see Section II.B.6 and Figures 5 and 6) added considerable potential confusion to the interpretation of data obtained with whole antiserum. Because of this, simplified methods were devised for purifying serum antibodies by eluting them from liposomes onto which they had been specifically bound via liposomal antigens.<sup>23</sup> With the method given below, starting only with antiserum and the ingredients for making liposomes, antiglycolipid antibodies can be obtained from whole antiserum within 4 h.

The steps are summarized as follows:

- (1) Liposomes + antiserum  $\xrightarrow[\text{wash}]{\text{centrifuge and}}$  LA (liposome-antibody)
- (2) LA + eluting solution  $\longrightarrow$  L + A
- (3) L + A  $\xrightarrow[\text{remove liposomes}]{\text{centrifuge to}}$  A (in eluting solution)
- (4) A in eluting solution  $\xrightarrow[\text{eluting solution}]{\text{dialyze to remove}}$  purified antibodies

Detailed properties of liposomes in the LA and LAC states are described elsewhere.<sup>29</sup>

#### B. PROCEDURE

Liposomes containing a glycolipid antigen are prepared as described above, except that 0.154 M NaCl is substituted for 0.308 M glucose as a swelling solution. Before use as an antibody source, rabbit antiglycolipid antiserum is heated at 56°C for 30 to 60 min to inactivate the complement. If the serum is lipoidal, or cloudy, or if there is even a remote possibility that it contains any precipitate, it should be clarified by centrifuging at  $27,000 \times g$  for 10 min at room temperature. The liposomes and antiserum (generally 0.4 ml of liposomes will remove all of the antiglycolipid antibodies from 1 ml of antiserum) are incubated together at room temperature for 30 min. The agglutinated liposomes that form (LA) are illustrated elsewhere (Figures 2 and 3 in Reference 29). Upon centrifugation ( $27,000 \times g$ ), the antiserum is

sufficiently dense that the agglutinated liposomes generally will float on the surface and the subnatant can be easily removed with a needle on a syringe, or with a Pasteur pipette.

The LA is washed by centrifugation in 0.15 *M* NaCl. Because of the attached protein, relatively low centrifugal force is adequate to form a tight pellet. A volume of eluting solution equivalent to the original volume of antiserum is added directly to the LA pellet. The pellet is dispersed with the aid of a Pasteur pipette, or with a syringe fitted with a 25-gauge needle. Elution may be performed as often as desired.

In most cases, 1 *M* NaI is a suitable eluting solution. The density of this solution is sufficiently great that, upon centrifugation, most of the liposomes float. This is important because, as the antibodies separate from the liposomes, the liposomes become progressively more difficult to centrifuge into a pellet. The subnatant is carefully removed with a syringe fitted with a 26-gauge needle.

A more drastic eluting reagent consists of equal volumes of chloroform and 0.15 *M* NaCl.<sup>23</sup> Upon phase separation, the lipids are dissolved in the chloroform and the antibodies in the saline. This mixture has the advantage of not requiring further dialysis to remove eluting solution. This method allowed elution of anti-Forssman antibodies that were not eluted by 1 *M* NaI.<sup>26</sup>

An alternative eluting agent used by Uemura et al.,<sup>25</sup> from which the liposomes were removed as a pellet, consisted of 2.5 *M* NaCl/0.01 *M* BaCl<sub>2</sub>. This reagent removed antibodies against sulfatide that were not eluted by either 1 *M* NaI or chloroform/saline.<sup>25</sup> Obviously, if the purified antibodies are to retain antigen binding activity after elution from liposomes they must be removed from the eluting solution, usually by dialysis.

The final purified antibody solution often contains a slight cloudiness due to contaminating liposomes. However, the liposome contamination usually is so slight that it is ignored. We have always found that any attempt to clarify the solution further by filtration through Millipore filters results in the loss of purified antibody activity.

### C. ADVANTAGES OF THE TECHNIQUE

This is a rapid, convenient method that can purify antibodies as much as 3800-fold directly from whole serum.<sup>23</sup> The degree of purification varies, depending on the antiserum. Approximately 50% of the original antibody activity in antiserum can be recovered. In a typical set of experiments, with one cycle of antibody binding and elution, approximately 75% of the recovered protein was antibody, and virtually all of the contaminating protein was removed by a second cycle of antibody binding to fresh liposomes followed by reelution.<sup>23</sup>

## IV. LIPID A

### A. BACKGROUND

Lipid A is the lipoidal constituent of lipopolysaccharide (LPS) from Gram-negative bacterial endotoxin. LPS has been referred to as a "super antigen" because only very small amounts are required to induce an immune response.<sup>42</sup> The reason for its great potency as an immunogen is that it is an antigen (polysaccharide) that carries its own adjuvant (lipid A). Upon incorporation into liposomes, lipid A is such a powerful adjuvant that it can even induce antibody production against the liposomes themselves.<sup>15-17</sup> By using lipid A as an adjuvant, monoclonal antiliposome antibodies can be obtained.<sup>18-21</sup> It is an outstanding adjuvant for inducing humoral immunity against liposome-associated protein antigens.<sup>7-14</sup> Lipid A itself can also serve as an excellent antigen, and, upon incorporation into liposomes, it readily reacts with anti-lipid A antibodies.<sup>8,15,43-45</sup> Lipid A is easily obtained from LPS, and it is not a single molecule. It actually consists of a heterogeneous mixture of compounds having similar structures.<sup>45</sup> The methods ordinarily used to obtain lipid A can result in the production of a "crude" or "impure" preparation. The crude contaminants of lipid A can be removed by treatment with ethylenediaminetetraacetic acid (EDTA) and chloroform extraction, resulting in purified lipid A that is chloroform soluble.<sup>44,45</sup>

In this section, we describe the preparation of lipid A, its purification by treatment with EDTA followed by chloroform extraction, and its incorporation into liposomes.

### B. MATERIALS

#### 1. LPS

LPS, prepared by either phenol-extraction (Westphal) or trichloroacetic acid treatment (Boivin) of Gram-negative bacterial cells, can be purchased from Calbiochem (San Diego, CA), Difco (Detroit, MI), List Biological Laboratories, Inc. (Campbell, CA), and Sigma (St. Louis, MO). Sigma also offers LPS from butanol-extracted cells.

#### 2. Lipid A

Lipid A can be obtained from Calbiochem, List Biological Laboratories, and Ribi Immunochem Research, Inc. (Hamilton, MT). The lipid A ("detoxified endotoxin") from Ribi is claimed to be less toxic (as tested by lethality for chick embryos) than standard endotoxin.<sup>46</sup>

### C. PROCEDURES FOR PREPARATION OF LIPID A AND INSERTION INTO LIPOSOMES

#### 1. Preparation of Lipid A

Lipid A is very easily obtained from LPS. The LPS is simply heated in a boiling water bath for 2 h in 1% acetic acid (10 mg of LPS per milliliter). The formed precipitate is washed three times with distilled water by centrif-

ugation (at 4°C) and lyophilized. The yield (wt/wt) for lipid A from *Escherichia coli* 0111 LPS (phenol-extracted, from Difco) is approximately 12 to 14%. The chloroform solubility of lipid A obtained in this way may vary from batch to batch of LPS, and this may be influenced both by the culture conditions of the original bacteria and the methods used to purify LPS.

## 2. Chloroform-Soluble Lipid A

Heavy metals and other polycationic contaminations interfere with the solubility of lipid A.<sup>47</sup> In order to purify lipid A, and to increase the fraction of chloroform-soluble lipid A, a Bligh-Dyer extraction in the presence of EDTA is performed as described by Dancey et al.<sup>44</sup> This can be done according to the scheme shown in Figure 7. An aqueous solution of 0.5% triethylamine (TEA) is used for solubilization of lipid A in the water phase. Since the lipid A may not all dissolve immediately, the solution is allowed to stand for 10 to 30 min.

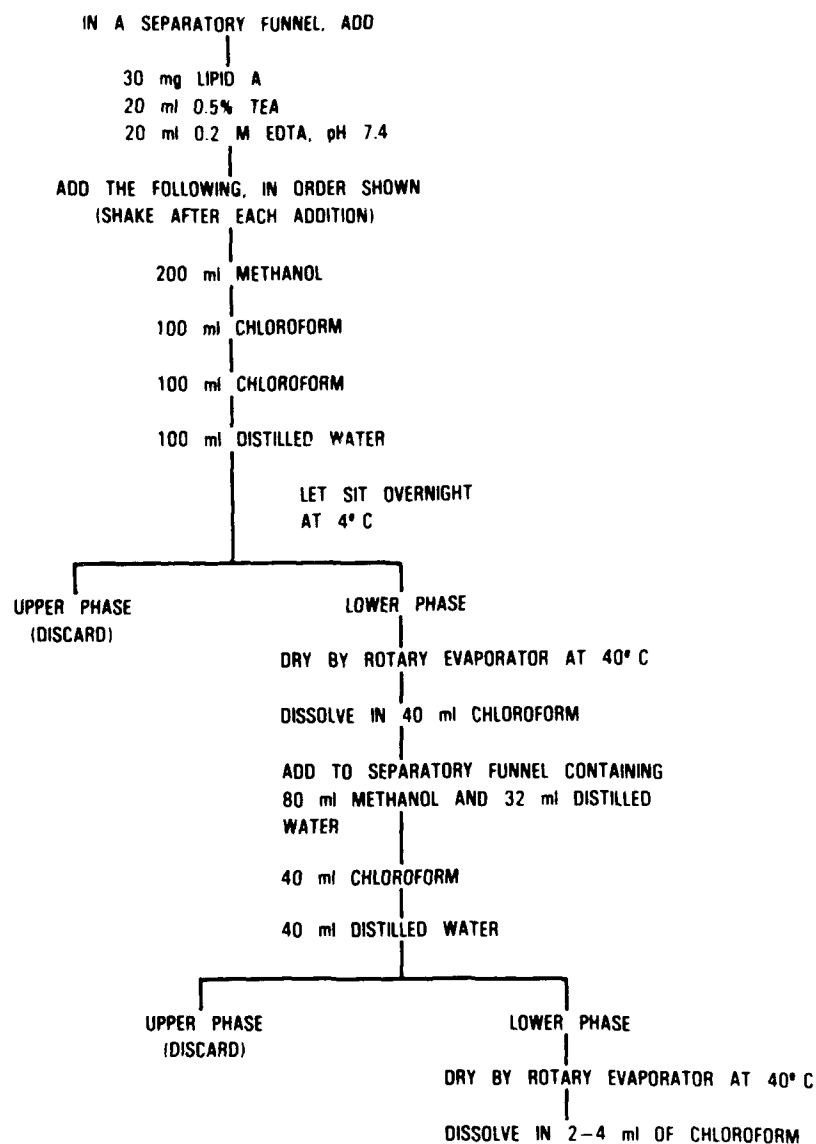
The separation of phases in the last step may require centrifugation (12,000  $\times$  g, for 10 min). The resulting purified lipid A in chloroform is analyzed for phosphate content.<sup>48</sup> For comparative purposes, we found that 1  $\mu$ g of chloroform-soluble lipid A from *E. coli* 0111 LPS (Difco) contained approximately 0.3 nmol of phosphate and 1  $\mu$ g of lipid A from *Salmonella minnesota* R595 (List) contained approx. 0.9 nmol of phosphate. Dancey et al.<sup>44</sup> found for *Salmonella* lipid A that 1  $\mu$ g contained approximately 0.7 nmol of phosphate. Most probably the phosphate to weight ratio will vary depending on the bacteria strain. Lipid A from List and Ribí are both chloroform soluble, but a small amount of methanol may be added to the chloroform to make the lipid A from List completely clear.

## 3. Insertion of Lipid A Into Liposomes

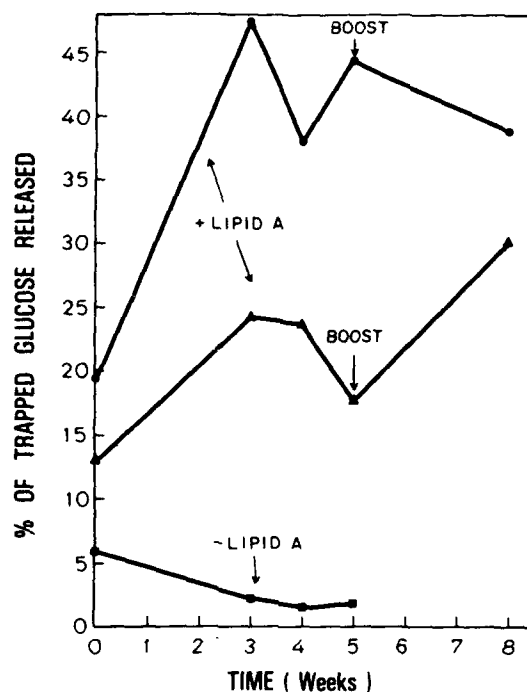
The materials and procedure for preparing liposomes are described above (Section II). Here we describe at which step chloroform-soluble or TEA-solubilized lipid A should be added, and the quantities of lipid A needed.

The chloroform-soluble lipid A is added to the pear-shaped flask together with the other lipids used for making up the liposomes (DMPC, Chol, DCP), and all of the lipids are taken to dryness together. The procedure for swelling follows the one earlier described (see Section II.B.1).

For purposes of immunization, a concentration of 20 nmol of lipid A phosphate/ $\mu$ mol of phosphatidylcholine (DMPC) has been found to be effective in giving rise both to antibodies against liposomes (e.g., Figure 8) and to antilipid A antibodies. For purposes of comparison it is worth noting that 12  $\mu$ g of native lipid A (*S. minnesota* R595) per  $\mu$ mol of phospholipid and 32  $\mu$ g of monophosphoryl lipid A (Ribí) per  $\mu$ mol of phospholipid each correspond to 20 nmol of lipid A phosphate per  $\mu$ mol of phospholipid. When tested by the glucose release assay, natural antiliposome antibodies often can be seen in the preimmunization bleedings of rabbits (but are less common in mice) and preimmunization bleedings are recommended.



**FIGURE 7.** Purification of lipid A using Bligh-Dyer extraction in the presence of EDTA. Note that dried lipid A is more readily solubilized in TEA prior to adding it to the separatory funnel.

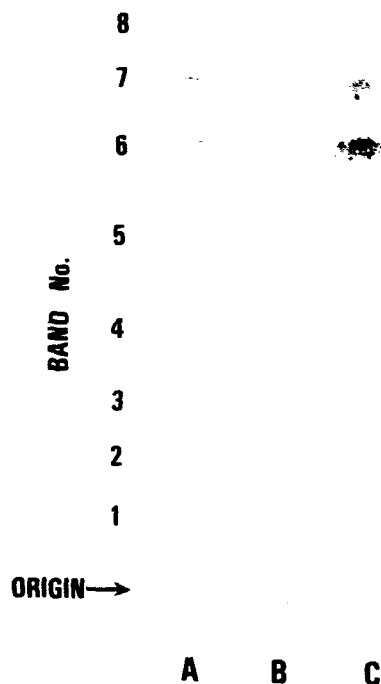


**FIGURE 8.** Rabbit antiliposome response. Two rabbits were immunized s.c. with liposomes containing DMPC/Chol/PI (0.89:0.75:0.11) plus 22  $\mu\text{g}$  of TEA-solubilized lipid A per  $\mu\text{mol}$  of PC. The total amount of lipid A injected was 0.22 mg. One rabbit was immunized with liposomes lacking lipid A. For the glucose release assay, the liposomes consisted of DMPC/Chol/DCP (1:0.75:0.11).

For purposes of analyzing antilipid A antibodies by the glucose release assay, a concentration of 2 nmol of lipid A phosphate per micromole of DMPC usually is adequate.<sup>45</sup>

For utilization in liposomes of lipid A that has not been purified by chloroform extraction, the lipid A is solubilized with 0.5% TEA (5 mg/ml) and added to a pear-shaped flask. It is then dried under nitrogen, with chloroform added to facilitate drying.<sup>15</sup> Then the chloroform solutions of the other liposome constituents are added to the flask and dried. The procedure of swelling follows the one described above in Section II.B.1. After inserting lipid A by this technique, suitable concentrations of lipid A for immunization and for glucose release assay are 50 and 20  $\mu\text{g}$  of lipid A, respectively, per micromole of DMPC.<sup>15</sup>

Recently, we have used different concentrations of lipid A and different types of lipid A in liposomes, and have reported some very interesting observations. Native lipid A from *S. minnesota* R595 (List) and monophosphoroseryl lipid A (Ribi) were 214-fold and 25-fold less pyrogenic than free lipid



**FIGURE 9.** Comparison of lipid A prepared in our laboratory (from *Salmonella minnesota* R595, lane A) with two commercially available lipid A preparations. *S. minnesota* R595 lipid A from List Biological Laboratories (lane B), and *S. typhimurium* lipid A from Ribi Immunochem. Research (lane C). The chromatograms were run on a silica gel 60 plate (0.25 mm, E. Merck, Darmstadt, Germany), developed with chloroform/methanol/water (130/45/7) as solvent, and stained for phosphate with molybdenum blue. The bands are numbered as described in Reference 45.

A, respectively, when incorporated into liposomes.<sup>12</sup> Accordingly, it is possible to use much higher concentrations of lipid A in liposomes for immunization than 20 nmol of lipid A phosphate per  $\mu\text{mol}$  of phospholipid without any adverse effects. We now generally use 250 to 350  $\mu\text{g}$  of lipid A per dose when immunizing rabbits with liposomes.

#### D. HETEROGENEITY OF PURIFIED LIPID A

Purified lipid A has a consistently heterogeneous pattern by thin layer chromatography.<sup>45</sup> Comparison of our lipid A with commercial preparations (also chloroform soluble) shows similar heterogeneity (Figure 9). The List preparation has all of the same bands found in ours, but in different amounts. The Ribi preparation is similarly heterogeneous, but it lacks all of the slow moving bands (corresponding to bands 1 to 4 in Reference 45) (Figure 9).



## V. PREPARATION AND USE OF LIPOSOMES FOR IMMUNIZATION

### A. PREPARATION OF LIPOSOMES

Liposomes used for immunizations are essentially prepared as described in Section II with some modifications. Stock solutions of lipids of known molarity in chloroform or chloroform-methanol (2:1, v/v), stored at  $-20^{\circ}\text{C}$ , are used. In most of our immunization schemes, the liposomes are composed of DMPC, DMPG and Chol in a molar ratio of 0.9:0.1:0.75 or DMPC, Chol, DCP in a molar ratio of 1.0:0.75:0.11. The liposomes also contain lipid A as an adjuvant in a concentration that will give a 40 to 50  $\mu\text{g}$  of lipid A/kg per injection. This dose corresponds to 20 nmol of lipid A phosphate per  $\mu\text{mol}$  of phospholipid when the lipid A source is *E. coli* 0111, and when the final phospholipid concentration is 10 mM. The 40 to 50  $\mu\text{g}$  of lipid A/kg dose was chosen because this was lower than the highest dose of liposomal lipid A that was nonpyrogenic when tested in rabbits by the standard pyrogenicity test.<sup>12</sup> For purposes of comparison, it is worth noting that 12  $\mu\text{g}$  of native lipid A/ $\mu\text{mol}$  phospholipid and 32  $\mu\text{g}$  monophosphoryl (MP) lipid A each corresponded to 20 nmol lipid A phosphate/ $\mu\text{mol}$  phospholipid.

Recently we have used different concentrations and different types of lipid A in liposomes and reported some very interesting observations. Native lipid A from List and MP lipid A from Ribi were 214-fold and 25-fold less pyrogenic, respectively, when encapsulated in liposomes.<sup>12</sup> Accordingly, from a theoretical standpoint, it might be possible to use much higher concentrations than 20 nmol of lipid A phosphate/ $\mu\text{mol}$  phospholipid without any adverse effect. We now generally use 100  $\mu\text{g}$  of lipid A/injection dose for immunization purposes in rabbits.

All of the liposomal components are mixed in a pear-shaped flask and dried in a rotary evaporator as described above. The dried lipid film is further desiccated overnight under very high vacuum ( $<10\ \mu\text{m Hg}$ ) in a desiccator. The liposomal lipids are then hydrated at 30 to 40 mM phospholipid concentration in sterile pyrogen-free water by shaking until all the lipid film is off the walls of the flask. The aqueous liposomes are then transferred to lyophilizer bottles and lyophilized. The lyophilized liposomes are reconstituted with the desired antigen in Dulbecco's phosphate buffered saline (DPBS) lacking  $\text{CaCl}_2$  and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (GIBCO Laboratories, Grand Island, NY) at 100 to 200 mM phospholipid concentration. The rationale for hydrating the liposomes in water, lyophilizing, and then dissolving in the antigen solution at high phospholipid concentration is to increase the percent of antigen encapsulated and, thereby save on the amount of antigen needed. At this step, antigen-reconstituted liposomes are washed to remove unencapsulated antigen by dilution with DPBS (about 20-fold) and centrifugation at  $29,000 \times g$ . The supernatants are aspirated and the liposomal pellets are resuspended in DPBS at a concentration of 20 to 100 mM phospholipid, depending on the

encapsulation efficiency. The amount of antigen encapsulated is then determined by a modified Lowry protein assay as described below.

## B. ANTIGEN ENCAPSULATION

### 1. Type of Antigen

The method described in Section V.A for preparing liposomes for immunization can be used with a variety of antigens. Protein antigens, and even unconjugated peptides, can be used in aqueous solutions to hydrate the lyophilized liposomes. The encapsulation efficiency generally varies between 25% and 40%, but may be as low as 10% or as high as 80%, depending on the peptide or protein. When one of a variety of recombinant proteins containing 32 tetrapeptide repeats derived from the circumsporozoite protein of *Plasmodium falciparum* sporozoites is used as an antigen, addition of 5 to 10 mg/ml solutions of antigen to the lyophilized liposomes will yield up to an approximate 1 mg/ml concentration in the washed and resuspended liposomes. With some unconjugated peptides,<sup>14</sup> it is possible to add the peptide solution directly to the dried lipid film and omit the lyophilization step.

When lipid antigens are used, it is not necessary to use the lyophilization/rehydration step of the procedure. The lipids that are dried as described in Section V.A in round bottom (or pear-shaped) flasks are hydrated in phosphate-buffered saline or 0.154 M NaCl, and it is presumed that 100% of the lipid antigen will be in the liposomal membrane. Lipid antigens are added to the liposomes at a concentration of either 100 nmol or 100  $\mu\text{g}/\mu\text{mol}$  of phospholipid.

### 2. Determination of the Amount of Encapsulated Protein Antigen

Some protein antigens, such as recombinant proteins containing 32 tetrapeptide repeats derived from the circumsporozoite protein of *P. falciparum*, cannot be measured by the standard Lowry procedure described previously<sup>23</sup> for liposomal lipids. Apparently these hydrophobic proteins are too tightly attached to the lipids to be measured.

The procedure for measuring encapsulation of such proteins involves two additional steps at the beginning of the Lowry assay. The samples are pipetted into 13  $\times$  100 disposable test tubes, 0.5 ml of chloroform is added to each tube, and the samples are evaporated to dryness in a SpeedVac Concentrator with the heater turned on. If the sample size is greater than 300  $\mu\text{l}$ , the sample volume is reduced by concentration in a SpeedVac Concentrator before addition of chloroform. The tubes can be stored covered with parafilm at 4°C for up to a week at this stage. To each tube of dried sample is added 0.2 ml of 15% sodium deoxycholate (which has been twice recrystallized), and the tubes are mixed on a vortex mixer at the highest speed for 20 to 30 s to ensure that all of the protein in the sample is redissolved. It should be noted that the sodium deoxycholate can be used without recrystallization, but the blank  $A_{750}$  readings are higher, and the solubility is decreased so that only a

13.5% solution can be used. At this point, Lowry reagent C should be added to the tubes, and the standard Lowry procedure should be completed as soon as possible, because after approximately 30 min the sodium deoxycholate solution of sample sometimes can become thick like a gel.

### C. USE OF ALUM AS AN ADDED ADJUVANT

The antibody titers obtained after immunization with some liposome-encapsulated proteins, although higher than with free antigen, are not as high as would be needed for an effective vaccine. Adsorption of liposomes containing encapsulated *Plasmodium falciparum* sporozoite antigens to alum just before immunization resulted in higher antibody titers than in the absence of alum.<sup>11,12</sup> In addition, the adsorption to alum results in the antibody titers remaining at high levels for greater than one year.<sup>11</sup>

Liposomes are prepared as described in Section V.A. Just prior to immunization, the liposomes are mixed with alum (aluminum hydroxide, Rehsorptar adsorptive gel, Armour Pharmaceutical Co., Kankakee, IL). The alum is received as a concentrated stock suspension at approximately 10 mg of aluminum per ml. This stock suspension settles out on storage and must be resuspended by mixing on a rotary shaker for 5 h. The desired volume is removed from the stock suspension after mixing and is diluted to 1.2 to 1.8 mg of aluminum per ml with phosphate buffered saline (PBS), pH 6.5 to 7.0.

For immunization of rabbits (NZW, 1 to 2 kg), 0.25 ml of liposomes containing encapsulated antigen are mixed with 0.25 ml of the diluted alum (typically diluted 1/1, v/v), and mixed vigorously by vortex mixer or by hand-shaking. The resulting 0.5 ml of liposome-alum suspension is injected intramuscularly (i.m.), with 0.25 ml injected into each haunch. For immunization of mice, 0.05 to 0.1 ml of liposomes containing encapsulated antigen are mixed with 0.1 ml of the diluted alum. The resulting liposome-alum suspension is injected i.m., typically with 0.1 ml injected into each haunch. For immunization of rhesus monkeys, 0.5 ml of liposome-encapsulated antigen is mixed with 0.5 ml of diluted alum. The resulting 1.0 ml is injected i.m.

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